PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/569, 33/535, C12Q 1/06 C12Q 1/68, 1/70 — A1

(11) International Publication Number: WO 91/00047

(43) International Publication Date: 10 January 1991 (10.01.91)

(21) International Application Number: PCT/US90/03717

(22) International Filing Date: 29 June 1990 (29.06.90)

(30) Priority data:
373,458 30 June 1989 (30.06.89) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94700 (US).

(72) Inventors: GRUBER, Harry, E. ; 13083 Maritime Place, San Diego, CA 92130 (US). JOLLY, Douglas, J. ; 3050 H Via Alicante Drive, San Diego, CA 92037 (US). KWANG, Hwei-Sing ; 605 Dockside Cove, Hastings, NB 68901 (US).

(74) Agents: DEVEZA, Robert, R. et al.; Knobbe, Martens, Olson & Bear, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RETROVIRUS DETECTION

(57) Abstract

A method, and groups of cells and retroviruses useful in the performance of the method, for testing for the presence or amount of a first retrovirus in a specimen. A replication defective retroviral vector construct having a marker gene sequence is exposed to the specimen and the presence or amount of a recombinant or pseudotyped retrovirus carrying the marker gene sequence is tested for. The replication defective vector may be disposed in cells, with the specimen being tested for the presence or amount of a retrovirus, or it may be disposed in a retrovirus, with the specimen being a group of cells which are tested for the presence or amount of said first retrovirus. In either case, where a first retrovirus complements the defective retroviral vector construct, a recombinant or pseudotyped retrovirus carrying the marker gene sequence will be produced. The first retrovirus to be tested for may be of a previously known, or unknown type, and may be any of a group of retroviruses.

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo			SN	Senegal
CH	Switzerland	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
DE	Germany, Federal Republic of	LK	Sri Lanka	TG	Togo
DK	Denmark	LU	Luxembourg	US	United States of America

RETROVIRUS DETECTION

Field of the Invention

This invention relates to retroviruses. It further relates to a method of testing for the presence of a retrovirus in a specimen, typically a previously unknown retrovirus, and to techniques of testing for rheumatoid arthritis based upon the presence of a retrovirus.

Rights of the U.S. Government

This invention was made with U.S. Government support under Grant Nos. AR-13622-18 and NS-21590-01AZ awarded by the Department of Health and Human Services. The U.S. Government has certain rights in this invention.

Background of the Invention

Retroviruses are single-stranded RNA viruses. Upon infection of a cell by a retrovirus the retroviral RNA genome is transcribed into its corresponding double-stranded DNA by a reverse transcriptase enzyme which is coded for by the viral genome. This DNA then enters the nucleus and integrates into the host DNA using an integrase enzyme which is also coded for by the viral genome. The integrated viral DNA ("proviral DNA") becomes a component of the host genome, replicating with it and being passed on to daughter cells in a stable manner. Cells carrying the proviral DNA are referred to as containing the retrovirus in the proviral form. The proviral DNA is comprised essentially of the gag, pol and env genes which are transcribed into viral RNA molecules and translated into the major viral proteins required for assembly of virus particles (the "packaging proteins"). The full length RNA transcript of the virus can be packaged by the viral packaging proteins into a viral particle which then buds from the cell enclosed within a portion of the cell membrane in which are embedded the viral envelope polypeptides. This membrane-coated viral particle is a fully competent viral particle which can infect other permissible cells.

The genome of a retrovirus (in either the RNA or DNA form) can be divided conceptually into two parts. The first, or "trans-acting" portion consists of the regions coding for

viral proteins. The second or "cis-acting" portion consists of regions of the viral genome necessary for replication and packaging. The trans-acting portion includes the group specific antigen ("gag") gene coding for synthesis of the core coat proteins, the "pol" gene coding for the synthesis of various enzymes (such as reverse transcriptase), and the envelope ("env") gene coding for the synthesis of envelope glycoproteins. Other proteins may also be produced in different retroviruses from messages produced by various internal splicing reactions. These viral functions determine a considerable part of the host specificity of a virus. In the murine leukemia virus ("MuLV") family, for example, the env gene products interact with cell surface receptors and determine whether the virus is ecotropic (infects only mice and rats), xenotropic (infects non-mouse species only), or amphotropic (infects mouse and other species, including human), and it has been suggested that the host range of a virus can be altered by replacing its env protein (see Milewski, Recombinant DNA Tehcnical Bulletin, Volume 9, Number 2, page 88 (1986)). The gag gene products also define the host-cell-range specificity in mice with respect to two main types: N (NIH derived mice) or B (Balb/c derived mice).

In general, the second part of the retoviral genome is referred to as the "cis-acting" portion which consists of the regions which must be in the genome to allow its replication and packaging into viral particles. These include the packaging signal, that identifies the viral RNA molecule as one to be packaged (ie. encapsidated) by viral proteins (ie. packaging proteins); 3' and 5' Long Terminal Repeat Sequences (3' and 5' "LTRs") with promoters and polyadenylation sites; and, two start sites for reverse transcription. The promoters, enhancers, and other regions of the LTRs are also capable of conferring tissue specificity such that the virus will only be "expressed" (i.e., transcribed or translated) in specific cell types even though it may infect others.

It has been recognized that the cis-acting elements are grouped at either end of the viral genome, in or near the

LTRs. Thus, the internal or "trans-acting" part of the provirus genome might be replaced by a gene of choice using routine techniques (Maniatis, T. et.al. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, 1982) to create a cloned "vector construct" (see for example, F. Ledley, The Journal of Pediatrics, vol. 110, No. 1, p. 1 (January, 1987)). Such a vector construct is defective in trans-acting functions but when the vector construct is placed in a cell where the necessary viral packaging proteins are supplied in trans-acting, the transcribed vector construct RNA should be packaged into viral particles which, in turn, will bud from the cell membrane. These "vector construct" particles will be indistinguishable in appearance from native "wild-type" virus particles, although they carry only the RNA of the vector construct. The vector construct particles can infect cells and the vector construct RNA can be carried into those cells where it will be reverse transcribed and subsequently integrated into the cell genome. It is believed that the gene will then be functional in the new cell but, without the trans-acting portion of the viral genome, it will be incapable of expressing the proteins required for viral replication and packaging. Hence, the vector construct and the vector construct virus particles are "replication defective" (also referred to herein simply as "defective"), and meaning that such vectors or virus therof are normally unable to produce new viral particles in the cell. The vector construct can, however, be transcribed and can express its gene product.

Retroviruses are known to be wide spread in non-human species and to cause various pathogenic conditions in animals. However, only a very small number of retroviruses have been identified in humans, and these only recently. In addition, new retroviral sequences have been detected in cells of human origin (see Callahan et.al., Science, Vol. 228, p. 1208, (1985)), although these sequences are generally not complete (i.e., not all of the retrovirus genome is present) and their significance remains unknown.

The cause of many human pathogenic conditions remains

unknown. One such pathogenic condition is human rheumatoid arthritis ("RA"). In various studies, viruses have been found to be present in body fluids from some RA patients, who were studied as part of a larger group of RA subjects. However, the results in such studies have been inconclusive in that, typically, a majority of the subjects did not exhibit the presence of the virus identified in the minority. For example, parvovirus has been demonstrated in RA synovial fluid in a minority of RA suffering patients. (Annals of The Rheumatic Diseases, Vol. 46, p.219-223, (1987)). Epstein-Barr ("EB") virus infection also appears to be present in a majority of patients with RA, but in no higher percentage than in the normal population, although RA patients are apparently less able to regulate such infections than normal control subjects (Bardwick et.al., Arthritis Rheumatism, Vol. 23, p. 626, (1980)). A retrovirus has been identified by Brassfield et.al., Arthritis Rheumatism, Vol. 25, p. 930 (1982), as the apparent cause of caprine arthritis, a goat arthritis clinically similar to human rheumatoid arthritis.

Previously, the standard techniques used in an effort to determine the presence of an unknown retrovirus (e.g. one whose presence may be suspected but which has not previously been isolated or characterized) have been the reverse transcriptase assay, electron microscopy, and various immunological and nucleic acid hybridization assays. Such techniques have been described, for example, by Weiss, R. in RNA Tumor Viruses, Vol. I, p. 209-260, (1982), Cold Spring Harbor Laboratory, New York.

"Complementation assays" have been used to isolate and identify replication defective animal retroviruses from cells. These defective animal retroviruses are known (i.e., they have been isolated and characterized previously and their defects are known to involve a missing or inoperative function), and "complementation assays" involve: a) infecting a cell carrying the defective animal retrovirus with a wild-type virus which supplies (i.e., "complements") the missing or defective function (referred to as "complementation"); and, b) assaying

for a biological property or attribute of the isolated (i.e., "rescued") defective animal retrovirus so that its presence can be identified. Complementation may comprise genetic recombination between the defective retrovirus nucleic acid gene sequence and the wild-type virus, or complementation of trans-acting defective viral functions (e.g., packaging proteins). Complementation assays have been used to determine the ability of a known rescued defective retrovirus to exert a specific biological effect. For example, in the S+ L- assay a murine leukemia virus rescues a replication defective Moloney sarcoma virus (MSV). The rescued-MSV infects an untransformed cell line and induces a transformation event in which the growth of the cells becomes uncontrolled in tissue culture and/or cell morphology becomes altered (Eckner and Kettrick, J. of Virology, Vol. 24, p. 383-390 (1979). It will be appreciated that being able to identify the rescued defective retovirus will depend upon the frequency of the rescue event in a population of cells, and that wild-type viruses which "optimally complement" the defective retrovirus will produce rescued retroviral particles at a higher frequency than those which do not. It will further be understood that where complementation involves genetic recombination, optimal nucleic acid sequence homology between the defective retrovirus and the wild-type virus will insure a higher frequency of rescue of defective retrovirus than if nucleic acid sequences are non-homologous. Such assays are time consuming, are highly dependent on the ability of the wild-type virus to appropriately complement the missing function and the ability of the recipient cell to become transformed. Measurements of the transformed state are subjective in view of the necessity to assess cell morphology and/or growth associated with the transformed state. In addition, these assays deal with known, characterized animal retroviruses and are used simply to isolate and identify those viruses. Complementation assays have not been used previously for unknown defective human retroviruses because the defective viral functions are unknown and the appropriate human

retrovirus useful in the complementation assay has also been unknown. The present invention recognizes selected retroviral vector constructs, (rather than wild-type virus), useful for isolating and identifying unknown human retroviruses at a detectable frequency.

As pointed out by Norval et.al., Ann. Rheum. Dis., Vol 38, p. 507 (1979) and Hart et.al., Ann. Rheum. Dis., Vol. 38, p. 514 (1979), the presence of a retrovirus in a majority of patients with RA has not been demonstrated, despite extensive efforts to determine the cause of RA. The failure to detect a retrovirus which is clearly associated with RA, or retroviruses associated with other human pathogenic conditions of unknown etiology, could be the result of relatively insensitive assays, i.e., the reverse transcriptase assay and electron microscopy, and the limitations of immunologic and nucleic acid hybridization assays which only search for viral proteins or nucleic acid sequences which are closely-related to those already known to exist. Further, many retroviruses can only grow in a few specific cell types, making it still more difficult to detect previously unknown retroviruses. For example, it is known that human T-cell lymphotrophic viruses types I and II (HTLV I and HTLVII) can only be effectively grown in certain cells such as in T-cell growth factor-driven lymphocytes, or cell lines derived therefrom, as described by Broder et.al., Ann. Rev. Immunol., Vol. 3, p. 321 (1985).

Retroviral constructs (i.e. retroviruses carrying vector constructs) such as those described by Gruber et.al. Science, Vol. 230, p. 1057-1061 (1985), including pLPLM, have been developed as vehicles for gene replacement therapy. The plasmid pLPLM, a murine leukemia virus-derived construct, has retroviral LTR promoters and packaging signals, but the pol, gag and env genes have been deleted and replaced by cDNA for human hypoxanthine phosphoribosyltransferase ("HPRT"). A.D. Miller et.al., Proc. natl. Acad. Sci. USA, ("PNAS"), Vol. 80, p. 4709-4713 (1983); J.K. Yee, Gene, Vol. 53, p. 97-104. It is known that when retroviral vector constructs having the LTRs, packaging signal, and a gene of interest, are placed in a cell

having a retroviral proviral genome expressing trans-acting portions of the genome, but lacking in cis-acting portions of the genome required for replication, such a "helper cell" or "packaging cell" will package RNA transcribed from the retroviral vector construct having a packaging signal, into a retroviral particle. Packaging of retrovirus genomes and retroviral vector constructs into viral particles can also occur when the retroviral proviral genome in the packaging cell is non-homologous with the retrovirus or retroviral vector construct, e.g. when the packaging cell contains integrated MuLV genes and the retroviral vector construct to be packaged is MSV or an avian or human virus. In this case the viral particle which is produced will contain an RNA from one virus packaged by the packaging proteins encoded by the non-homologus proviral genome. Such virus are said to be "pseudotyped" or to be a "pseudotype" of the original virus, and this process is referred to as "pseudotyping" a virus. In a complementation assay, virus rescue can occur either through genetic recombination or complementation of trans-acting functions, i.e. packaging of a defective retrovirus into infective viral particles with packaging proteins. If the trans-acting functions are supplied by a non-homologous virus, the rescued defective retrovirus would be said to be a "pseudotype" and the process may be referred to a "pseudotyping", to distinguish the process as a restricted form of a complementation assay. For additional clarification see, e.g., PCT Application No. WO 86/00922, published February 13, 1986; Molecular Biology of Tumor Viruses, Second Edition, "RNA Tumor Viruses", Robert Weiss (Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA (1984); Mann et.al., Cell, Vol. 33, p. 153-159 (May 1983); Watanabe et.al., Molecular and Cellular Biology, Vol. 3, No. 12, p. 2241-2249 (Dec. 1983); Watanabe et.al., Proc. Natl. Acad. Sci. USA, Vol. 79, p. 5986-5990 (October, 1982); and Watanabe et.al., Eukaryotic Viral Vectors, p. 115-121, Y. Gluzman (Ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, US (1982). Pseudotyping has been used to rescue replication defective

retrovirus in cases where the wild-type virus used in the rescue, was comprised of a nucleic acid sequence which was not homologous with the defective retrovirus, although the frequency of such rescue events is appreciably lower than the frequency observed with an optimally homologous wild-type rescue virus. This appreciably lower frequency may still be detectable since it will occur at a rate which is 0.1%-1% of the frequency observed with an optimal wild-type rescue virus. For example, see Linial, J. Virology, Vol. 38, No. 1, p.380-382 (April, 1981). Since an optimal wild-type rescue virus can produce in its own right 10^6 infectious virus units/ ml, the frequencies described above (i.e. 0.1%-1%) for rescued defective retrovirus will be easily detectable.

Summary of the Invention

The present invention provides a method of testing either laboratory or clinical specimens for the presence or amount of a retrovirus (sometimes referred to herein as a "first retrovirus"). The specimen, or portion thereof, is brought into contact with a defective retroviral vector construct which carries a marker gene sequence. The vector construct is carried by either an infectious retrovirus or by a cell (i.e., the proviral form of the vector construct). The first retrovirus may be either a known or previously unknown retrovirus (an "unknown retrovirus" being one which has not previously been isolated and characterized). The specimen can be a fluid sample from cells or tissues, such as tissue culture medium, serum or plasma, or it can be cells. In the method described and claimed herein a replication defective retroviral vector construct containing a marker gene sequence is used to test for the presence of a first retrovirus, in a specimen. If the first retrovirus is present the retroviral vector construct is complemented and an infective replication incompetent retrovirus will be produced which carries the marker gene sequence. The presence of the marker in a pseudotyped recombinant retrovirus will indicate that the specimen contained an unknown retrovirus which was capable of complementing the replication defective retroviral vector

construct to give an infective replication incompetent retrovirus. The marker gene sequence is selected so that its presence can be conveniently determined. The replication defective retroviral vector will be one with a packaging signal, a marker sequence, and other required sequences (generally the 3' and 5' LTRs) so that when inserted into a cell, and in the presence of the requisite integrase enzymes, the defective vector construct can be integrated into the cell's genome and express the marker gene. The defective retroviral vector construct may optionally code for additional other proteins, including any of the gag, pol, or env proteins, required to produce a complete but replication incompetent retrovirus carrying the retroviral vector with the marker gene. However, it will be understood that the defective retroviral vector construct is "defective" in the sense that it will not contain one or a plurality of cis-functions or trans-functions which are normally required for production of infectious retroviral particles.

In the practice of the invention a specimen is brought into contact with either test cells (i.e., comprising a retroviral vector construct with a marker gene sequence integrated in the proviral form), or alternatively, with a test medium (i.e., comprising medium having an infective retrovirus carrying the defective vector construct with the marker gene sequence). If the specimen contains a defective first retrovirus, and if the retroviral vector construct in the test cells or test medium is able to complement that defective function (i.e., of the first retrovirus), then pseudotyped infective retrovirus may be produced which will carry the marker gene sequence. It will be understood that by assaying for the presence of the marker gene sequence, a positive assay result will indicate the presence of a retrovirus in the original specimen. The assay may be biological (e.g., confering resistance to a toxic drug to the cells infected with the pseudotyped retrovirus), enzymatic (e.g., wherein the marker gene sequence encodes a marker enzyme), immunochemical (i.e., wherein the marker gene

sequence encodes a marker antigen which can bind an antibody), or alternatively, may involve identifying the pseudotyped retrovirus nucleic acid in the cell by hybridization techniques (i.e., Maniatis, op.cit.), or other methods known to those skilled in the art.

It will be understood that where the specimen contains a previously unknown and uncharacterized replication defective retrovirus, it may not be possible to fully anticipate the retroviral vector construct which will optimally complement the defective function(s). In this case, rescue events will occur at a low frequency and it may be necessary to conduct multiple tests on multiple specimens using relatively large amounts of specimen material and relatively large volumes of tissue culture medium obtained from incubation of the test cells with the specimen. It may also be necessary to conduct the assays for the marker gene using less diluted samples, as will be evident to those skilled in the art. In contrast, where the specimen contains a previously known and characterized retrovirus, it will be possible to make a retroviral vector construct which optimally complements the missing defective function, thereby increasing the relative frequency of rescue events and making identification of the marker gene sequence less difficult.

In a preferred case, whether the first retrovirus is of a type which was previously known or previously unknown, a defective retroviral vector construct can be designed which will be "optimally complementing", i.e. comprising a defective retroviral vector construct designed to code for all packaging proteins except one. In this case the assay is said to be a "minimal complementation" assay because the unknown first retrovirus must supply only one (i.e., "minimal") function in order to produce infective virus particles. In a minimal complementation assay the chances of detecting the first retrovirus in the specimen may be increased, since the first retrovirus need only provide a "minimum complementation", i.e. it need supply only the one function missing in the defective retroviral vector construct.

In an extension of the preferred embodiment, different portions of the specimen may be independently brought into contact with a plurality of different defective retroviral vector constructs comprising a group of vector constructs, wherein each of construct of the group has a marker gene sequence and a sequence coding for a corresponding packaging protein different from the packaging protein coded for by the sequences of the other remaining vector constructs in the group, and all of the vector constructs together in the group have the sequences which code (in aggregate) for all the packaging proteins required to rescue the first retrovirus and produce an infective recombinant retrovirus identifiable by the presence of a marker gene sequence. In this manner it will be possible to find a suitable retroviral vector construct within the group which complements the defective first retrovirus in the specimen. In the case where the defective first retrovirus in the specimen is deficient in more than one function, it will be understood that two or more vector constructs of the group may be added to portions of the specimen provided that these vector constructs do not complement one another and thereby produce an irrelevant recombinant infective retroviral particles. A single specimen may also be tested with different groups of vector constructs, by way of example (but not limited to) a first group comprised of vector constructs derived from MuLV, a second group of vector constructs derived from Rous sarcoma retrovirus (RSV), or a third group of vector constructs derived from mink cell focus forming (MCF) retrovirus. By comparing either the amount of recombinant retrovirus produced (e.g., viral titer or level of marker gene) in the different groups of vector constructs, and by different members within a group, it will be possible to identify a retroviral construct which is complementary to the defective first retrovirus in the specimen and thereby to increase the frequency of the rescue event so that unknown first retroviruses can be identified.

In another preferred embodiment of the invention, test cells (i.e., cells which carry the replication defective

retroviral vector construct in the proviral form), are cultured with the specimen. In this embodiment the first retrovirus in the specimen may not have been previously isolated or characterized, but it may be infective for the test cell, or alternatively, its nucleic acid may enter the test cell (e.g., through transfection or other means). Such a specimen may also be comprised either of a fluid sample containing retrovirus, or alternatively of cells.

Complementation of the defective retroviral vector construct integrated in the test cell may occur either by recombination with the first retrovirus nucleic acid or as a result of trans-acting functions which can be supplied if the first retrovirus integrates into the test cell genome and expresses packaging proteins which are capable of rescuing and thereby pseudotyping the defective retroviral vector construct ("direct complementation"). Alternatively, "indirect complementation" may result from activation of gene expression by an exogenous agent (e.g., IL-1, IL-2 or other protein or virus) capable of activating retrovirus gene expression from the proviral form of the first retrovirus integrated in the test cell genome. It will be appreciated that the specimen can be cells ("specimen cells") to be tested for a first retrovirus and that, in such cases, for example, the defective retroviral vector construct can be introduced into those specimen cells by appropriate means (i.e., by infection, transfection, electroporation, or other means known to those skilled in the art).

If a first retrovirus is present in the specimen and the test cells are susceptible to infection by it, recombinant or pseudotyped infective retrovirus particles may be produced and enter the tissue culture medium; wherein these retroviruses will carry a marker gene sequence encoded initially by the retroviral vector construct. The presence of recombinant or pseudotyped retrovirus in these samples can be identified by collecting the tissue culture medium and adding it to a second group of test cells ("secondary test cells"). The secondary test cells may be the same or different from the initial test

cells, and may be selected for their ability to become infected by the recombinant retrovirus, as described above. If the secondary test cells are susceptible to the recombinant retrovirus, they will become infected, and the marker gene will become incorporated into the genome of the secondary test cells. The presence of the marker gene sequence in the secondary test cells is then assayed to identify the presence of the retrovirus in the specimen.

In the case where the method is used to assay for the presence of a particular type of known and characterized retrovirus in a specimen, test cells can be chosen which are known to be susceptible to infection by that particular type of retrovirus. Likewise, secondary test cells would be chosen which are: a) known to be susceptible to infection by the possible recombinant or pseudotyped retrovirus which could be produced from the test cells; and/or , b) capable of expressing the marker gene sequence.

Various marker gene sequences can be chosen. However, the marker which will be expressed in the test cells (or secondary test cells) is preferably a gene sequence encoding a protein selected from: (i) an enzyme; (ii) a protein which will enable the test cells (or secondary test cells) to grow in a medium in which they would not otherwise grow (e.g.(but not limited to), in the presence of a toxic drug, or in the presence of a selective media deficient in certain key factors required for cell growth); and, (iii) an antigen and, in particular, one which will be expressed on the cell surface. The presence of an expressible marker gene sequence in the test cells (or secondary test cells) is identified by assaying for the marker gene sequence transcription or translation product. For example, the marker gene sequence may code for HPRT and secondary test cells will be selected which are deficient in HPRT. The secondary test cells incubated with media from the test cell culture will be infected with recombinant or pseudotyped retrovirus containing the HPRT gene and this will enable the secondary test cells to grow in hypoxanthine-aminopterine-thymidine (HAT) selective medium. If the

secondary test cells grow in the HAT medium, this will be an indication that the specimen contained a retrovirus. Of course, other marker gene sequences can be used, e.g., the gene for β-galactosidase or luciferase.

Where a specimen is to be assayed for an unknown retrovirus, or assayed for the presence of any of a group of known retroviruses, it can be useful to choose the test cells (or secondary test cells) from a plurality of different cell lines. As earlier described, a retrovirus can be highly specific as to which cells it will infect and which cells will allow expression of the retroviral gene products. Thus, in the case of testing a specimen for an unknown retrovirus, using a plurality of different cell lines increases the chances that a retrovirus, if present in the specimen, will productively infect the test cells and express the marker gene sequence.

It is further preferable that the secondary test cells are not from the same cell line as the test cells, and that microscopic (and/or other) morphological differences may exist in order to distinguish between the two cell types in a tissue culture containing both cell types. A situation might exist where tissue culture medium collected from the test cell culture was contaminated with test cells and when it was added to the secondary test cell culture some test cells would also be inadvertently added. If the presence of contaminanting test cells can be identified in the secondary test cell culture it may prove possible to remove them by exposing the culture to a selective medium which will permit growth of the secondary test cells while killing the contaminating test cells. This is an advantage because it helps eliminate potential false positive results which could be associated with the growth of test cells (potentially containing marker gene sequences integrated in the proviral form) in the secondary test cell cultures.

In the case where patient cells are used as the "test cells", (i.e., retroviral vector constructs are introduced directly into the patient cells), it is considered preferrable to use secondary test cells of the same type as the patient

cells (e.g., lymphocytes if the patient cells are lymphocytes, or fibroblasts if they are fibroblasts, etc.) since this will improve the chances that any pseudotypes or recombinant retrovirus produced in the patient cells will infect the secondary test cells. It will be recognized that non-malignant cells in patient samples have a limited capacity to grow in tissue culture and if they inadvertently contaminate a tissue culture of secondary test cells, they will eventually become numerically inferior to the secondary test cells in such a culture.

Generally, another technique which will increase the chances of detecting an unknown retrovirus in a specimen is to provide different packaging signals in the retroviral vector construct having the marker gene sequence. Thus, an unknown retrovirus in a specimen may be able to form a recombinant retrovirus pseudotype at a low frequency with a first retroviral vector construct having a first packaging signal, but it may be able to form a pseudotype at a higher frequency with a second retroviral vector construct having a different (second) packaging signal.

In the case where test cells are used which contain the replication defective retrovirus vector construct in a proviral form, a further technique to increase the chances of detecting the first retrovirus in a specimen (and particularly if it is of an unknown type), is to select a plurality of test cells forming a group which is comprised of individual members having different retroviral vector proviral genomes and wherein each member of the group contains a proviral genome coding for at least one packaging protein, and further, each member of the group codes for a different packaging protein (i.e., gag, pol or env). For example, the proviral genome of one member of the group of test cells could code for a retroviral vector construct having a marker gene sequence, another member could code for the env protein, and still another could code for gag protein. This arrangement will increase the chance that recombinant retroviruses can be produced from the test cells upon exposure to a specimen

containing an unknown retrovirus, since the chances for complementation are increased. In a "minimal complementation" arrangement the genome of the test cells has a retroviral vector construct integrated in proviral form that has a marker gene sequence and all the sequences required to produce infective recombinant or pseudotyped retrovirus virus particles, except one. In such a minimal complementation arrangement, the first retrovirus in the specimen need only supply the one missing function (i.e., gene or protein) in the test cells in order to form the recombinant or pseudotyped retrovirus having the marker gene sequence. Thus, the chances of producing retrovirus particles carrying the marker gene sequence (i.e., recombinant or pseudotyped) may be increased.

A means for confirming or identifying the presence of a first retrovirus in a specimen, where the first retrovirus is of a previously known and characterized type, is to culture test cells with portions of the specimen independently in the presence and absence of antibody specific for the known retrovirus. Neutralization and/or inactivation of the retrovirus by the antibody will result in a reduced number of secondary test cells having the marker gene sequence encoded by the recombinant or pseudotyped retrovirus, as compared with secondary test cells not incubated with the antibody.

Further steps which may be used with the above methods include a "wash step", in which the test cells are collected as a centrifugal pellet in the bottom of a centrifuge tube, the supernatant tissue culture medium is discarded, and the cells are placed back into tissue culture with fresh tissue culture medium. Preferably the washing step is conducted on the test cells about one day prior to collecting a sample from the test cell culture to be used with secondary test cells. The washing step is useful for reducing the levels of inactive recombinant retrovirus, (i.e., recombinant retrovirus which may bind to cellular receptors but are damaged in some manner so that they cannot productively infect the secondary test cells). In this way damaged retroviral particles are eliminated and do not interfer with infection of the secondary

test cells by the infective recombinant or pseudotyped retrovirus particles. The presence of such damaged retrovirus could reduce the sensitivity of the assay for identifying recombinant or pseudotyped retrovirus. Further, the test cells or secondary test cells, or both, can be treated to enhance their ability to be infected with any recombinant retrovirus, e.g., with a detergent.

The present invention also provides test cells and secondary test cells which are useful in the above detection methods.

Using the above detection methods, the inventors have found an association of a positive assay result with specimens from patients fulfilling the diagnostic criteria of rheumatoid arthritis. This apparent retrovirus (or potentially group of retroviruses or a member of such a group) is referred to throughout this application as "rheumatoid arthritis associated retrovirus" (RA retrovirus), or equivalent expressions. As a result of this discovery, the present invention also provides for the testing of rheumatoid arthritis in a human subject, which requires testing for the presence of rheumatoid arthritis associated retrovirus in the subject using methods of the present invention.

Brief Description of the Drawings

Embodiments of the invention will be described with reference to the drawings, in which:

Figure 1 illustrates a Moloney MLV genome with various deletions which can be made thereto, the resulting genomes being useful in a minimum complementation assay described below (see Example 5, below);

Figure 2 illustrates an amphotrophic envelope expression vector, useful to produce an amphotrophic envelope protein for a recombinant retrovirus carrying a marker sequence (see Example 5, below).

Detailed Description of the Preferred Embodiments

Fluid specimens from a number of human subjects have been evaluated for the presence of a retrovirus using the methods of the present invention. Test cells from various cell lines

were used, including SV-40 transformed fibroblasts derived from a Lesch-Nyhan child (LNSV), which were transfected with pLSΔPΔLM (the resulting recombinant cells being referred to as the LNΔP line) and selected for the HPRT-containing phenotype. pLSΔPΔLM is a plasmid carrying a murine leukemia virus-derived vector construct with LTRs and packaging signals, but without pol, gag, and env genes, which were removed and replaced by the cDNA for the mutant HPRT. pLSΔPΔLM is the same as pLPL2 (Miller & Buttimore, Mol. Cell. Biol., Vol. 6, p. 2895-2902 (1986)), but with the HPRT gene mutated as described by Yee et.al., Gene, Vol. 53, p. 97-104 (1987)). Another primary cell line used was an EB virus transformed lymphoblast line derived from a Lesch-Nyhan child (cell line "1547"), which cells were infected with a replication incompetent HPRT-containing retrovirus ("LPL") and selected for the HPRT-containing phenotype. The preparation of such cells is described by Willis et.al., J. Biol. Chem., Vol. 259, p. 7842-7849 (1984). The foregoing cell line does not spontaneously produce HPRT-containing retroviruses. A third cell line used as a test cell source consisted of normal human cultured bone marrow, infected with a high titer preparation of retrovirus containing the pLPLM vector construct from the producer line 7A2, as described in Gruber et.al., supra Science (1985).

Secondary test cells should be selected which are likely to be susceptible to infection by a recombinant retrovirus which may be produced from the test cells. The secondary test cells should preferably be infectable by the retrovirus which is used as the retrovirus used in constructing the vector carrying the marker gene sequence. In the trials below, cell lines used as secondary test cells in the assays were the HPRT-deficient rat 208F or murine B77 cells (with regard to these cell lines see, respectively, Miller et.al., PNAS, Vol. 80, p. 4709-4713 (1983) and Miller et.al. Molec. Cell. Biol., Vol. 5, p. 431-437, (1985)). Other cells used as secondary test cells included human HPRT-deficient lymphoblasts (1547), fibroblasts (LNSV), or cultured human bone marrow cells. In the last case the first retrovirus in the specimen is exposed

to human bone marrow cells. The recombinant or pseudotyped retrovirus resulting from this test cell can be "re-pseudotyped" by: a) infecting a secondary test cell with the pseudotyped retrovirus; b) infecting the same test cells for a second time ("superinfected") with a murine amphotropic retrovirus (MA) (Miller et.al., supra. Mol. Cell. Biol., (1985)); and, c) collecting the tissue culture medium from the superinfected secondary test cell culture and using it to infect 208F or B77 cell lines ("tertiary test cells"). The advantage of this procedure is that the final progeny recombinant re-pseudotyped retrovirus from the secondary test cells will be known to be infective for the tertiary test cells.

The procedure followed in testing the specimens was generally as follows. Test cells containing the pLSΔPΔLM or vector construct were cultured with the specimen to be tested. After 3 to 5 days, supernatant from the foregoing culture was placed onto an HPRT-deficient secondary test cell line and after 24 hours the cell line was grown in HAT selection medium. The secondary test cell is preselected (prior to use) in 6-thioguanine every four weeks to remove any HPRT-containing revertants. The test cells are maintained in tissue culture medium containing HAT as a precautionary measure until shortly before use to ensure retention of the HPRT vector in the cells. When supernatants from LNΔP are tested on secondary test cells such as 208F or LNSV, a negative control (i.e., 208F or LNSV cells alone) is included in the experiment to test for the possible presence of spontaneous HPRT+ revertants in the secondary test cell cultures. The cultivation of the LNΔP cells for 3 days after adding PBLs, and before transferring the supernatant to secondary test cells is a minimum time, but longer cultivation (up to 30 days) also allows detection of recombinant retrovirus containing the HPRT marker gene sequence in the secondary test cells, and sometimes this longer time was accompanied by a larger signal (i.e. greater expression of the marker gene). Thus, when setting up the assy it is optimal to utilize a time course,

e.g., 3,7,11,14,21, and 30 days at which times the supernant tissue culture medium is collected and transferred from the test cells to the secondary test cells.

Control experiments were performed simultaneous with the preceding steps, wherein tissue culture medium from untreated test cells was transferred to secondary test cells to assure a) that the test cells are not contaminating the medium sample being transferred to the secondary test cells, and b) that the test cells are not spontaneously producing a recombinant retrovirus carrying the marker gene sequence. This control procedure also assures that the secondary test cells have not developed spontaneous HPRT+ revertants. Positive controls were also run, in which the test cells were exposed to the MA retrovirus, and the resultant recombinant retrovirus in the tissue culture medium was transferred to the secondary test cells to assure that the retrovirus detection assay system was indeed functioning properly.

The secondary test cell was cultured for two weeks in HAT selective medium in tissue culture dishes, the dishes were washed, fixed in methanol, stained with Crystal violet, and the colonies were counted. Each colony represents one HPRT-containing recombinant or pseudotyped retrovirus. The titer of recombinant or pseudotyped retroviruses in the specimen was determined by serially diluting portions of the specimen to determine the dilution at which recombinant or pseudotyped retrovirus became undetectable in the assay ("limiting dilution assay").

In addition, further and improved sensitivity was possible using detergent to facilitate adherence of retrovirus to the test cell (or secondary test cell). Using the MA retrovirus positive control, the method was developed as follows. Polybrene detergent was added to the 208F cell line before infection with a tissue culture medium specimen containing MA retrovirus. The detergent facilitated membrane adherence and, therefore, increased the ease with which retroviruses infected the test cells (or secondary test cells). The 208F secondary test cells used in this protocol

were set up one day before they were treated with detergent and infected with the MA retrovirus specimen. The LNSV test cell line was exposed to the specimen and the test cells were washed the day before the tissue culture medium was removed from the culture and transferred to the 208F secondary test cells. The wash step removed inactive (non-infective) recombinant retroviral particles which could potentially interfer with binding of infective recombinant retrovirus particles to the secondary test cells.

EXAMPLE 1

On day one, 2 X 10^5 cells from the test cell line LNΔP were plated in DMEM plus 10% Fetal Bovine Serum (FBS) in 60mm tissue culture dishes. The cells were cultured in 10% CO_2/90% Air at 37°C. On day four, polybrene was added to the media to a final concentration of 4μg/ml and at least 2 hours later the specimen comprising peripheral blood lymphocytes (PBLs) was added. PBLs were prepared from patient or normal human blood samples collected into heparin as an anticoagulant, and the PBLs were processed rapidly (within 2 hours of being collected) on Ficoll/Hypaque (Histopaque-tm). The PBLs were washed extensively (at least 3 times) to remove platelets and 10^6 PBLs were added to each dish of LNΔP cells in a total of 4 ml DMEM containing 10% FBS. The positive control was 100μl of a viral supernatant of the MA murine amphotropic retrovirus, which was added to the LNΔP cells; the negative control (apart from the normal individuals) was LNΔP cells cultured with no additions. On day seven, the secondary test cells (either rat 208F cells or human LNSV cells), were established as cultures in a separate series of tissue culture dishes by placing 1-2 X 10^5 cells in a 60mm dish in αMEM containing 10% FBS and polybrene (4μg/ml). At least four hours later, the tissue culture medium was removed from the LNΔP test cell/PBL cultures and cells were removed by either filtration through a 0.45μ filter or centrifugation at 1500 rpm for 5 minutes in a clniical centrifuge. Media was removed from the secondary test cell culture dishes and 1-2 ml of the

clarified test cell/PBL tissue culture medium was added to each dish followed by 2ml of αMEM containing 10% FBS and polybrene (4μg/ml). These secondary cell cultures dishes were then incubated 24 hours in 5%CO_2/95% Air at 37°C. On day eight, the medium was changed to αMEM containing 10% FBS and HAT (10^{-4}M hypoxanthine, 2 X 10^{-7}M Aminopterin, 5 X 10^{-5} thymidine). The medium was subsequently changed every 3 days and on day 18 the culture dishes were washed, fixed, stained with Crystal violet, and the colonies were counted.

Using the above protocol, the first fifty-two clinical specimens examined included forty-nine synovial fluids, two synovial biopsies, and one blood sample. Most of the forty-nine fluids were from patients with diagnosed RA, while the remainder included specimens from patients with Reiter's syndrome, osteoarthritis, gout and a monoarticular chronic arthritis. Approximately two thirds of the RA specimens tested produced a positive result using the assay procedure described. One patient with Reiter's syndrome also tested positive, but had evidence of HTLV III infection as determined by nucleic acid hybridization assay. Utilization of the various different cell lines described, as test cells and secondary test cells, did not significantly alter the assay results. The titer of retrovirus in the synovial fluid specimens was relatively constant, in the range of 1 to 30 colonies per milliliter of synovial fluid.

Experiments also indicated that positive assay results were obtained more frequently with cellular materials, i.e., in specimen material that was associated with centrifugal pellets and not supernatants.

Another control experiment using Southern blot analysis confirmed that the pLSΔPΔLM retroviral construct is actually transferred from the LNΔP test cells to the secondary 208F cells. Specifically, DNA was isolated from the 208F cells after they had been incubated in the above procedure with patient specimens, and selected for HAT resistance. Fragments of this DNA were prepared using the Sst I restriction endonuclease, the DNA fragments were electrophoresed on

agarose gels, and the gel was assayed with a radioactive nucleic acid probe made from the pLSΔPΔLM vector (Maniatis, supra). An appropriate 4 kilobase (kb) fragment was detected in the 208F DNA indicating that the pLSΔPΔLM retroviral vector construct was integrated into the secondary test cell DNA.

A further experiment was conducted using PBLs from a patient diagnosed with RA. It was shown that such lymphocytes when infected with the replication defective pLSΔPΔLM vector are able to form recombinant retrovirus pseudotypes which infected 208F secondary test cells.

In a further experiment, patient PBLs (the same patient as those used in the example directly above) were incubated with test cells consisting of U937 cells carrying a retroviral vector construct termed "Bag", an acronym for β-galactosidase (Cepko, PNAS 84, 156-160 (1987)). The foregoing retroviral vector contains both the β-galactosidase gene and the drug resistance gene for neomycin. A second culture of the U937 cells (lacking the Bag vector) was used as the secondary test cells. As shown below in Table 2, the secondary test cells were found to carry the β-galactosidase gene.

EXAMPLE 2

Peripheral blood lymphocytes were isolated on four different occasions from one rheumatoid arthritis patient (designated patient WT), and three other patients and 5 normal individuals. The PBLs were co-cultivated iwth LNΔP test cells and after three (3) days the supernatants were transferred to the 208F secondary test cells and these cells were assayed for the presence of the vector construct by exposing them to HAT selective medium. Column 1 of Table 1 shows the control supernatants from LNΔP test cells cultured without patient or normal PBL. Column 2 shows the results of experimental cultures where patient or normal PBLs were cultured with the LNΔP test cells. Column 3 shows the results obtained in positive control experiments where patient or normal PBL were incubated with 1μl (for patient) or 10μl (for normal controls) of a test MA viral supernatant in the presence of the LNΔP test cells. As an additional positive control, Column 4 shows

the results obtained when 1μl or 10μl of MA viral test supernatant was added directly to the LNΔP cultures (i.e., in the absence of normal or patient PBLs). The results in Table 1 are expressed as the number of 208F secondary test cell colonies observed to be present after 10 days of culture in the presence of HAT selective medium. The experiments were performed in triplicate and the numbers in Table 1 indicate the results of the three assays (separated by commas). Negative controls in these experiments included 208F secondary test cells exposed to the HAT selective medium, and the results of these experiments were always completely negative (i.e., no colonies were observed after 10 days).

TABLE 1

RA Patients	LNΔP Sup	PBL on LNΔP	PBL+MA on LNΔP	MA on LNΔP	on LNΔP
WT	Expt.#1	00,00,00	12,07,16	05,07,06	14,09,07
	Expt.#2	00,00,00	06,01,02	04,00,06	03,10,07
	Expt.#3	02,00,00	04,07,01	03,09,05	09,15,02
	Expt.#4	00,00,00	05.03,06	06,05,10	10,10,05
CT	00,00,00	02,06,05	04,05,07	07,10,12	
WR	00,00,00	03,04,03	08,12,05	10,12,09	
JC	00,00,00	05,02,07	03,06,19	05,13,11	
Normals					
KT	00,00,00	00,00,00	04,--,--	01,--,--	
AG	00,00,00	00,00,00	68,82,63	--,100,92	
BS	00,00,00	00,00,00	37,45,26	40,39,67	
JD	00,00,00	00,00,00	17,06,--	108,21,38	
M	00,00,00	00,00,00	42,19,31	26,12,35	

EXAMPLE 3

The assay was carried out as in Example 2 but the PBLs were cocultivated with LNΔP cells for up to 14 days and the supernatants were transferred to the 208F secondary test cells at days 3 and 14 of the test cell culture. In this case the assay with the 14 day culture specimens was performed in the absence of the polybrene-pretreatment (-polybrene) of the secondary test cells, or in the presence of the polybrene-

pretreatment (+polybrene) (TNTC-= too numerous to count). The results are shown in Table 2, below.

TABLE 2

Time of test cell cocultivation:	3 days		14 days
	-polybrene	+polybrene	
LNΔP (negative control)	00,01,00	-- --	
MA (positive control)	TNTC	TNTC TNTC	
RA Patients			
1	00,01,00	28,09,01	13,00,--
2	00,00,00	01,02,01	03,01,00
3	00,04,00	01,03,02	01,16,20
4	01,00,00	02,01,01	03,00,01
5	00,00,00	00,02,00	00,03,05

EXAMPLE 4

Peripheral blood lymphocytes from an RA patient (WT) were isolated on Ficoll Hypaque density gradients and 5 X 10^6 cells were cultivated in RPMI 1640 medium (containing 10% FBS and Phytohemagglutinin (PHA)) with 10^4 cfu of defective retroviral Bag vector construct (Price et.al., PNAS 84: 156-160 (1987)), having been shown previously to contain less than 1 unit of replication competent virus per ml. After 3 days, 1 ml of supernatant tissue culture medium was removed from the culture and transferred to U937 test cells growing in the same medium. Twenty-four hours later the cells were fixed and assayed for the presence of β-galactosidase enzyme activity by histochemical methods wherein a positive test result is indicated by the presence of a microscopically-blue cell (Price et.al. supra) (Experiment #1, Table 3). Control cultures consisted of: 10^4 units of Bag vector added directly to the U937 test cells and the cultures assayed 24 hours later (positive control; Experiment #2); 10^8 units of MA virus added to the U937 test cells and the cultures assayed 24 hours later (negative control; Experiment #3); 10^8 units of MA virus and 10^4 units of Bag vector added to patient PBLs, cultivated for 3 days, and the supernatant tissue culture medium transferred

to the U937 test cells which were then assayed 24 hours later (Experiment #4). Other control experiments showed that 10^4 units of Bag vector incubated in medium at 37°C for 3 days was no longer infective (i.e., it did not produce blue-U937 cells). Thus any Bag retrovirus observed after 4 days in culture is recombinant or pseudotyped retrovirus and not the original Bag vector.

TABLE 3

Experiment	Blue Cells/10^5 U937 cells	
#1 (Bag+PBL) (on U937)	6	10
#2 (U937+Bag)	$>10^3$	$>10^3$
#3 (U937+MA)	0	0
#4 (Bag+PBL+MA) (on U937)	>100	--

The appearance of blue cells, even at a low level can be used to assay for the presence of a retrovirus in this quick (4 day) assay.

The complementation assay (as used in the foregoing examples) relies on an unknown retrovirus in a specimen providing either complementation of trans-acting functions (i.e., packaging proteins) for the Bag vector-MuLV retroviral vector constuct, or alternatively, the unknown retrovirus genetically recombines with the Bag vector to produce infective recombinant retrovirus. In either case, the pseudotyped or recombinant retrovirus (respectively) buds from the test cell (i.e., the PBLs) and infects a secondary test cell (i.e., the U937 cells), where the presence of the marker gene sequence (i.e., the β-galactosidase marker gene in the Bag vector) is assayed. In the case of the pseudotyped Bag retrovirus, the ability of the initial Bag retroviral vector construct to be packaged by the unknown retrovirus in the specimen depends on its ability to recognizing the packaging signal of the Bag vector, (albeit at a low frequency because of the large evolutionary distance between the two

retrovirus), and packaging it into infective pseudotyped retrovirus particles which can bud from the PBLs and infect U937 cells. To increase the chances that the unknown retrovirus in the specimen may successfully complement the retroviral vector construct, a minimum complementation assay was designed in which the retroviral vector construct is deficient in only one retroviral function: a particular example of which is provided in Example 5, below.

EXAMPLE 5

In this particular example of a minimum complementation assay, complementing defective retroviral genomes were designed which express all but one of the MuLV viral functions. These complementary genomes were transfected into the test cell line which already contained the retroviral vector construct having a marker gene sequence. Thus, the unknown retrovirus in the specimen has only to supply one missing function and the test cell will produce pseudotyped or recombinant retrovirus containing the marker gene sequence. It is important that the test cell used in this case (i.e., human LNΔP cells) is not a mouse cell, since mouse cells are known to be capable of repairing defective MuLV retroviral genomes. The overall effect of a minimum complementation assay is to a) make the assay more sensitive for detecting the presence of an unknown retrovirus in the specimen; and, b) to permit detection of a wider range of retrovirus types encompassing those with relatively poor ability to complement the MuLV retroviral vector construct.

A number of separate in frame deletions were made in the indicated domains of the cloned Moloney-MuLV genome designed pMLV-K (see Table 4 below and Figure 1). These deletions were prepared by restriction endonuclease digestion to remove fragments of the genome, or by site-directed oligonucleotide mutagenesis. Eight different defective MuLV genomes, containing different deletions, were prepared and each deletion is indicated in Table 4, below. The nucleotide numbers (occurring after the restriction sites in Table 4), refer to the nucleotide numbering of the MuLV genome as it

appears in "RNA Tumor Viruses", Vol. 2, 1985, Cold Spring Habor Laboratory. The methods for preparing the deletion mutants is well known to those skilled in the art (see Maniatis supra).

TABLE 4

Deletion Mutants of Moloney MLV (MuLV)

Mutant	Deletion Deletion Size	Exact Domain	AminoAcid Deletion bp	AminoAcid Deletion
1)p700	HindIII(4894)/SphI(5137)	Integrase	243	81
2)p800	SalI(3705)/HindIII(4894)	RT	1185	395
3)p900	EpaI(5816)/EpaI(7195)	Env	1380	460
4)p1100	HindIII(4894)/SacII(4949)	Integrase	54	18
5)p1200	By mutagenesis	Protease	201	67
6)p1600	BalI(1672)/BalI(2053)	p30	381	127
7)p1400	BglII(1906)/BstE2(2453)	p30,10,14	543	181
8)p1700	StaI(2689)/StaI(3622)	RT	933	311

An assay can then be performed using any of these modified defective MuLV retroviral vector constructs to prepare LNΔP test cell lines (by transfection), as previously described in the examples above. These LNΔP test cells can then be used in complementation assays, as described above. Alternatively, the defective Moloney MuLV retrovirus can be used to construct a defective retroviral vector construct which can be used directly to infect cells in a specimen, also as described above. The defective Moloney MuLV retroviruses, and their plasmids and vector constructs, can also be used to prepare test cells (or secondary test cells) from other suitable human cells or cells from other species. In another preferred embodiment, the defective Moloney MuLV genomes of the MLV-K strain (encoding an envelope protein with specificity for ecotropic cell receptors) are used to prepare a defective retroviral construct which is introduced into a test cell; and, the secondary test cell is selected from cells which are known to be susceptible to infection with the MLV-K (i.e., murine cells with the ecotropic virus receptor). Moloney MuLV is available from the American Type Culture Collection (Rockville, MD, USA) under deposit number ATCC VR-190, the MLV-K strain can be prepared from MLV as described by

Miller et.al. Molec. Cell. Biol., Vol. 5, p.431-437 (1985). Thus, the ecotropic envelope encoded in the test cell will "help" in this case to assemble a pseudotyped or recombinant retrovirus from the unknown retrovirus in the specimen, and the pseudotype or recombinant will have specificity for cells with ecotropic viral receptors. In the case of non-murine cell lines, it will be appreciated that test cells constructed with retroviral vector constructs encoding amphotropic envelope proteins will be capable of "helping" infection of cells with amphotropic viral receptors in a similar manner to the "help" provided by ecotropic envelope with murine cells.

Various modifications and alterations to the embodiments of the invention described above, can be envisaged by one skilled in the art. Accordingly, the present invention is not limited to the specific embodiments described herein.

WE CLAIM:

1. A method of testing for the presence or amount of a retrovirus in a specimen comprising:

(a) exposing a replication defective retroviral vector having a marker gene sequence to the specimen or a portion thereof such that the retrovirus can complement the replication defective retroviral vector construct and produce a recombinant or pseudotyped retrovirus carrying the marker gene sequence; and

(b) testing for the presence or amount of production of the marker gene sequence which will indicate the presence or amount of a recombinant or pseudotyped retrovirus.

2. A method as defined in claim 1 wherein the replication defective retroviral vector is a vector construct.

3. A method as defined in claim 1 wherein the replication defective retroviral vector is carried by test cells and the test cells are cultured with the specimen or a portion thereof.

4. A method as defined in claim 3 wherein the replication defective retroviral vector is in proviral form.

5. A method as defined in claim 1 wherein the replication defective retroviral vector is carried by a retroviral particle and the specimen comprises cells to be tested for a retrovirus.

6. The method as defined in claim 1 wherein the marker gene sequence codes for a protein selected from the group consisting of an enzyme, an antigen, and a protein which will permit growth of secondary test cells in a medium in which they would not otherwise grow.

7. The method as defined in claim 6 wherein said antigen is a cell surface antigen.

8. A method as defined in any of claims 1 through 7 wherein the replication defective vector also carries a gene sequence coding for at least one packaging protein.

9. A method as defined in any of claims 1 through 7 wherein the replication defective retroviral vector also

carries gene sequences coding for all the packaging proteins necessary for replication, except one missing packaging protein, so that the retrovirus being tested for need only produce the missing protein to produce the recombinant or pseudotyped retrovirus carrying the marker gene sequence.

10. A method as defined in any of claims 1 through 7 wherein a plurality of groups of replication defective retroviral vectors are exposed to respective portions of the specimen, the retroviral vectors of each group having a marker sequence and a sequence which codes for a corresponding packaging protein different from the packaging protein coded for by the sequences of the remaining groups, all of the groups together having gene sequences which code for all packaging proteins required to produce a recombinant or pseudotyped retrovirus carrying a marker gene sequence.

11. A method as defined in any of claims 1 through 7 wherein the presence or amount of a previously unknown retrovirus is tested for.

12. A method as defined in any of claims 1 through 7 wherein the presence or amount of a previously known retrovirus is tested for, and wherein the defective retroviral vector has at least one cis-acting gene sequence different from the known retrovirus being tested for.

13. A method as defined in claim 1 through 7 wherein the specimen is from a mammal.

14. A method as defined in any of claims 1 through 7 wherein the specimen is a clinical specimen.

15. A method of testing for the presence or amount of a retrovirus in a specimen, comprising:

(a) culturing the specimen or a portion thereof with test cells susceptible to infection with the retrovirus disposed in a test cell medium, which test cells carry a replication defective retroviral vector having a marker sequence, such that a retrovirus, if present, may complement the replication defective retroviral vector and produce recombinant or pseudotyped retrovirus carrying the marker gene sequence into the

test cell medium;

(b) culturing secondary test cells with at least a portion of the test cell medium under conditions sufficient to allow infection to occur; and

(c) testing for the presence or amount of the marker gene sequence in the secondary test cells, and therefrom determining the presence of retrovirus.

16. A method as defined in claim 15 wherein the replication defective vector is a vector construct.

17. A method of testing for the presence or amount of a known type of retrovirus in a specimen, comprising:

(a) culturing the specimen or a portion thereof with test cells disposed in a test cell medium, which test cells are susceptible to infection by the known type of retrovirus being tested for and which carry a replication defective retroviral vector with a marker gene sequence, such that the known type of retrovirus, if present in the specimen, can infect the test cells and complement the replication defective vector to produce in the test cell medium a recombinant or pseudotyped retrovirus carrying the marker gene sequence;

(b) culturing secondary test cells which are susceptible to infection by the recombinant or pseudotyped retrovirus with a least a portion of the test cell medium under conditions sufficient to allow infection to occur; and

(c) testing for the presence or amount of the marker gene sequence in the secondary test cells, and therefrom determining the presence of the retrovirus.

18. A method as defined in claim 15 or 17 wherein said marker gene sequence is selected from the group consisting of nucleic acid encoding a protein selected from an enzyme, a protein which will enable growth of the secondary cells in a medium in which they would not otherwise grow, and an antigen.

19. A method as defined in claim 17 or 18 wherein the defective retroviral vector has at least one cis-acting gene sequence corresponding to that of a retrovirus of a type

different from the retrovirus being tested for.

20. A method as defined in any of claims 3 or 15 wherein the presence or amount of a previously unknown retrovirus is being tested for and the test cells used are from a plurality of different cell lines.

21. A method as defined in any of claims 15, 17, or 18 wherein the replication defective retroviral vector also carries a sequence which can produce at least one packaging protein in the test cells.

22. A method as deinfed in any one of claims 15, 17, or 18 wherein the replication defective retroviral vector also carries sequences coding for all the packaging proteins necessary for replication, except one missing packaging protein, so that the retrovirus being tested for need only produce the missing protein in the test cells to produce the recombinant or pseudotyped retrovirus carrying the marker gene sequence.

23. A method as defined in any of claims 17 or 18 wherein the secondary test cells are not from any of the same cell lines as the test cells.

24. A method as defined in claim 5 wherein the specimen cells are cultured with a group of replication defective retroviruses each having a vector construct carrying a marker gene sequence and at least one gene sequence capable of producing a corresponding packaging protein, with at least one of the vector constructs coding for a packaging protein different from a packaging protein of the remaining vector constructs.

25. A method as defined in claim 5 wherein a plurality of groups of replication defective retroviruses are cultured with respective portions of the specimen cells, the retroviral vectors of each group having a marker sequence and a sequence which codes for a corresponding packaging protein different from the packaging protein coded for by the sequences of the remaining groups, all of the groups together having vector sequences which code for all packaging proteins required to produce a recombinant retrovirus carrying the marker gene

sequence.

26. A method as defined in any of claims 15 or 17 wherein the retroviral vector constructs carried by some of the test cells contain a different packaging signal from the retroviral vector constructs carried by other of the test cells.

27. A method as defined in any of claims 5, 24, or 25 wherein the vectors of some of the replication defective retroviruses differ in packaging signal from those of others of the replication defective retroviruses.

28. A method as defined in claim 17 further comprising the step of culturing additional test cells with a portion of the specimen and an antibody to the known retrovirus before culturing secondary test cells with a portion of the test cell medium, and testing for a reduced number of secondary test cells carrying the marker gene sequence as a result of the presence of the antibody.

29. A method as defined in claim 15 further comprising the step of washing the test cells and further culturing the test cells prior to culturing the secondary test cells with the test cell medium, so as to reduce the concentration of any damaged or inactive recombinant or pseudotyped retroviruses which may otherwise be present in the primary cell medium.

30. A method as defined in claim 15 additionally comprising treating at least one of the test and secondary test cells with a membrane active agent prior to culturing the test cells with the specimen to enhance the ability of the cells to be infected with a recombinant or pseudotyped retrovirus.

31. Test cells useful in testing for the presence or amount of a retrovirus in a specimen, which test cells have a genome coding for a replication defective retrovirus with a marker gene sequence.

32. A method of testing for rheumatoid arthritis in a human subject, comprising testing for the presence or amount of a rheumatoid arthritis associated retrovirus in the subject.

33. A method of evaluating the efficacy of a drug therapy for rheumatoid arthritis, comprising testing the effect of the drug on growth of rheumatoid arthritis associated retrovirus.

34. A method as defined in claim 1, 5, 6, or 15 wherein the retrovirus being tested is rheumatoid arthritis associated retrovirus.

35. A group of test cells useful in testing for the presence or amount of a retrovirus in a specimen, which test cells are from a plurality of different cell lines and have a genome coding for a replication defective retrovirus with a marker gene sequence.

36. A kit comprising a group of test cells or retroviruses, useful in testing for the presence or amount of a retroviral genome coding for a replication defective retroviral vector construct with a marker gene sequence, with some of the vector constructs coding for a packaging protein different from the packaging protein for which the vector constructs of others of the group codes.

37. A kit comprising a group of test cells or retroviruses, useful in testing for the presence or amount of a retroviral genome in a specimen, which test cells or retroviruses have a genome coding for a replication defective retrovirus with a marker gene sequence, wherein the genome of some of the test cells or retroviruses have a retroviral packaging signal which differs from the retroviral packaging signal possessed by the genome of others of the group.

38. A kit as defined in claim 37, wherein the test cells or retroviruses are test cells, the genome of which additionally codes for at least one of the packaging proteins required to produce retroviruses carrying the marker gene sequence.

39. A kit as defined in any of claims 36 to 38, wherein the marker gene sequence can express, in a secondary test cell infected by a recombinant retrovirus carrying the marker gene sequence, a protein selected from an enzyme, a protein which will enable growth of the secondary test cells in a medium in

which they would otherwise not grow, and an antigen.

40. A replication defective retroviral vector having a marker gene sequence and a gene sequence coding for at least one packaging protein.

41. A replication defective retroviral vector having a marker sequence and all of the sequences, except one missing sequence corresponding to a packaging protein, which are necessary to make, in a cell, replication competent retroviruses which carry the marker sequence.

42. A retrovirus carrying a replication defective retroviral vector as defined in claim 40 or 41.

43. A cell carrying a replication defective retroviral vector as defined in claim 40 or 41.

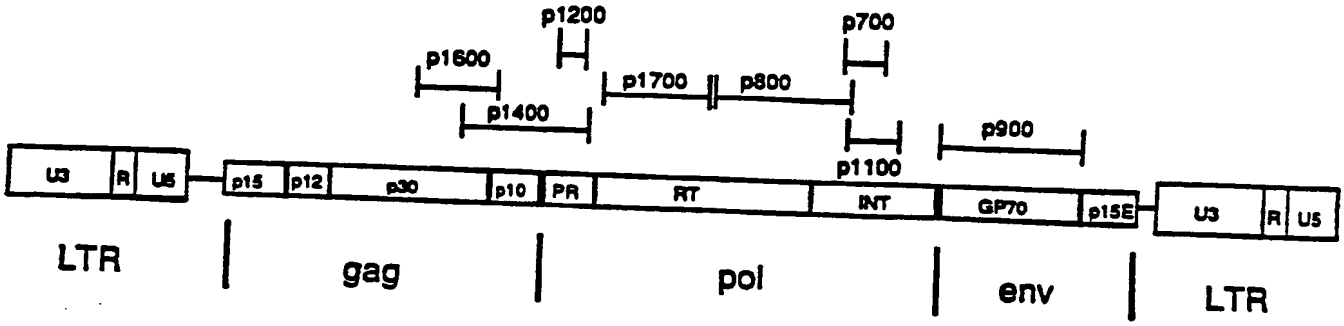
44. A Moloney murine lymphoid leukemia retroviral vector having a sequence corresponding to any one of the proviral forms thereof designated herein as p700, p800, p900, p1100, p1200, p1600, p1400, or p1700.

45. A cell carrying a Moloney murine lymphoid leukemia retroviral vector as defined in claim 44.

46. A retrovirus carrying a Moloney murine lymphoid leukemia retroviral vector as defined in claim 44.

Fig. 1

DELETION MUTANTS IN THE MLV GENOME
FOR THE MINIMAL COMPLEMENTATION ASSAY



2

Fig. EXPRESSION VECTOR FOR THE AMPHOTROPIC ENVELOPE GENE

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/03717

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 33/569,33/535; C12Q 1/06,1/68,1/70
US Cl.: 435/5,6,7,29,34,39,235,240.2,810,948

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System 5	Classification Symbols
US Cl.	435/5,6,7,29,34,39,235,240.2,810,948

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 6

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category * 15	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X,P Y,P	WO, A, 90/02797 (R. M. SHUMAN), 22 March 1990, see pages 12 and 13.	1-6,8,12,13,15-19,21,31,35-40,42,43 34 32,33 34
X,P Y,P	WO, A, 89/11285 (S. GAY) 30 November 1989, see pages 11 and 14.	
X	Journal of General Virology, Volume 67, part 7, issued July 1986, W. OSTERTAG ET AL., "The Myeloproliferative Sarcoma Virus Retains Transforming Functions after Introduction of a Dominant Selectable Marker Gene", pages 1361-1371 see abstract page 1361.	44-46

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 3	Date of Mailing of this International Search Report 4
17 October 1990	04 DEC 1990

International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	Christine V. Nucker